

Bacterial Nitric-oxide Synthases Operate without a Dedicated Redox Partner^{*[S]}

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Bacterial nitric-oxide (NO) synthases (bNOSs) are smaller than their mammalian counterparts. They lack an essential reductase domain that supplies electrons during NO biosynthesis. This and other structural peculiarities have raised doubts about whether bNOSs were capable of producing NO *in vivo*. Here we demonstrate that bNOS enzymes from *Bacillus subtilis* and *Bacillus anthracis* do indeed produce NO in living cells and accomplish this task by hijacking available cellular redox partners that are not normally committed to NO production. These “promiscuous” bacterial reductases also support NO synthesis by the oxygenase domain of mammalian NOS expressed in *Escherichia coli*. Our results suggest that bNOS is an early precursor of eukaryotic NOS and that it acquired its dedicated reductase domain later in evolution. This work also suggests that alternatively spliced forms of mammalian NOSs lacking their reductase domains could still be functional *in vivo*. On a practical side, bNOS-containing probiotic bacteria offer a unique advantage over conventional chemical NO donors in generating continuous, readily controllable physiological levels of NO, suggesting a possibility of utilizing such live NO donors for research and clinical needs.

Nitric oxide (NO)² signaling is essential in organisms ranging from bacteria to humans (1–6). In eukaryotes, NO is mainly synthesized by arginine-oxidizing NO synthases (eNOS). This enzyme functions as a homodimer and exists in mammals in three major isoforms: neuronal, endothelial, and inducible (3, 7). eNOS enzymes are expressed as a single polypeptide and share the common general organization in which the oxygenase and reductase domains are linked via a regulatory calmodulin-binding domain (Fig. 1) (7). The oxygenase domain contains heme at the active center that binds a substrate, arginine. The

reductase domain binds cofactors and provides electrons for arginine oxidation. The calmodulin-binding domain performs a regulatory function. Upon calcium-induced calmodulin binding, the reductase and oxygenase domains form a complex, allowing electrons to flow from NADPH to the active center for executing NOS chemistry (7). The latter is a multistep process. Iron in the resting enzyme exists in the ferric state. To activate the enzyme and initiate NO synthesis, Fe(III) has to be reduced to Fe(II). The reductase domain of eNOS enzymes performs this task by transferring electrons from NADPH via FAD and FMN. In the presence of tetrahydrobiopterin (BH₄), the enzyme then binds and oxidizes arginine in two steps to form NO (7, 8).

Genomic and functional analyses indicate that NOS-like enzymes are present in all kingdoms of life (Fig. 2) (9–13). At least one representative from plantae, protozoa, archaea, and bacteria have a NOS homolog, which underscores the evolutionary conservation of this enzyme and the importance of NO signaling in general (Fig. 2). Bacterial-derived NO has been solely attributed to denitrification. In this process, inorganic nitrite is converted to NO and then further to dinitrogen by action of several reductases (14). However, three phyla (firmicutes, actinobacteria, and deinococcus) of Gram-positive microorganisms have enzymes that are homologous to the oxygenase domain of eNOS (Fig. 2). There is a significantly high level of homology between bacterial and eukaryotic NOSs, with ~45% of amino acids being identical and 50–60% being similar (supplemental Fig. S1). Moreover, functional and structural analyses of isolated bacterial NOS-like proteins (bNOS) have demonstrated their high degree of similarity with eNOS (8, 15, 16). The bNOS active center also associates with a ferric iron-heme complex. As with eNOS, bNOS forms a dimer and facilitates arginine and N^ω-hydroxy-L-arginine oxidation in a multistep fashion (8, 10, 15, 16). Furthermore, purified bNOS successfully oxidizes arginine to NO *in vitro*, if provided with an electron donor such as H₂O₂ or a mammalian reductase domain (9).

Notably, all known bacterial and archaeal NOS enzymes lack a reductase domain, a feature that has raised doubts about their ability to produce NO *in vivo*. Recent reports, including one from our laboratories, provide evidence for bNOS-mediated NO production in living bacteria, but they do not address how the systems can operate without a reductase domain (17, 18). Bacterial genomes encode for many factual and hypothetical

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[S] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. S1 and Table S1.

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² The abbreviations used are: NO, nitric oxide; NOS, nitric-oxide synthase; eNOS, eukaryotic NOS; bNOS, bacterial NOS; nNOS, neuronal NOS; LB, Luria Bertani; WT, wild type.

reductases, which in principle could support bNOS activity *in trans*. For example, the YkuNP reductase from *Bacillus subtilis* has recently been proposed to be such a redox partner based on *in vitro* reconstitution experiments (19). In the present study, we provide further information concerning the production of NO via bNOS from *B. subtilis* and *Bacillus anthracis* *in vivo*. In particular, we show that bNOS enzymes are promiscuous with

respect to their redox partners and can utilize a nonspecific cellular reductase even in organisms that do not have their own bNOS. Such flexibility in choosing a redox partner by bNOS is remarkable from both a regulatory and an evolutionary perspective.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Arginine, arabinose, H_2O_2 , and MAHMA NONOate were from Sigma. The ligand (FL) for the NO-specific probe was synthesized as described previously (20, 21). The probe (CuFL) was generated *in situ* by mixing FL and $CuCl_2$ in a 1:1 ratio.

General Methods—*B. subtilis* and *Escherichia coli* overnight cultures grown in liquid Luria Bertani (LB) medium were diluted 1:100 in fresh LB and grown at 37 °C with aeration until the A_{600} reached 0.5, unless indicated otherwise. To induce arabinose-dependent promoters, 2% arabinose was used. Preparation of *B. subtilis* competent cells was carried out by the Spizizen method (22). Antibiotics were used at the following concentrations: chloramphenicol 5 μ g/ml, ampicillin 100 μ g/ml, and spectinomycin 100 μ g/ml. To determine H_2O_2 resistance, *B. subtilis* cells were exposed to 10 mM H_2O_2 for 30 min. The number of viable cells was determined by colony formation on LB agar. Colony-forming units were counted the following day and the percentage survival calculated.

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Strains and Plasmids—Strains used in this study are listed in Table 1. *B. subtilis* 168 (*trpC2*) was used as a parent strain. Plasmids were constructed using standard methods and amplified in *E. coli* Top 10 (Invitrogen). All PCR fragments were amplified from *B. subtilis* 168 and *B. anthracis* Stern chromosomal DNA using Phusion DNA polymerase (New England Biolabs). Oligonucleotide primers were purchased from IDT. To construct pBNOS_{B.su} and pBNOS_{B.an}, the *nos* gene from *B. subtilis* or *B. anthracis* was amplified by PCR and cloned into the pBADb expression vector (Invitrogen). To prepare pBNOS_{B.su}(I/V), we used oligonucleotide-directed mutagenesis (5'-AAAGACCTCGAGC-TTCATATCAGACACAATAG-GCAC-3', 5'-ATATGAAGCTCG-AGGTCGGGGGC-3'). The construction *B. subtilis* Δ *nos* strain was described previously (23). The same procedure was used to construct

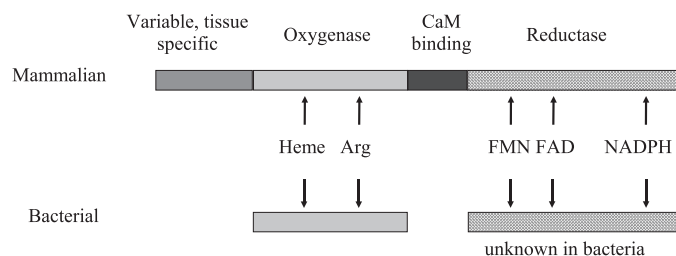


FIGURE 1. Comparison of mammalian and bacterial NOS structures.

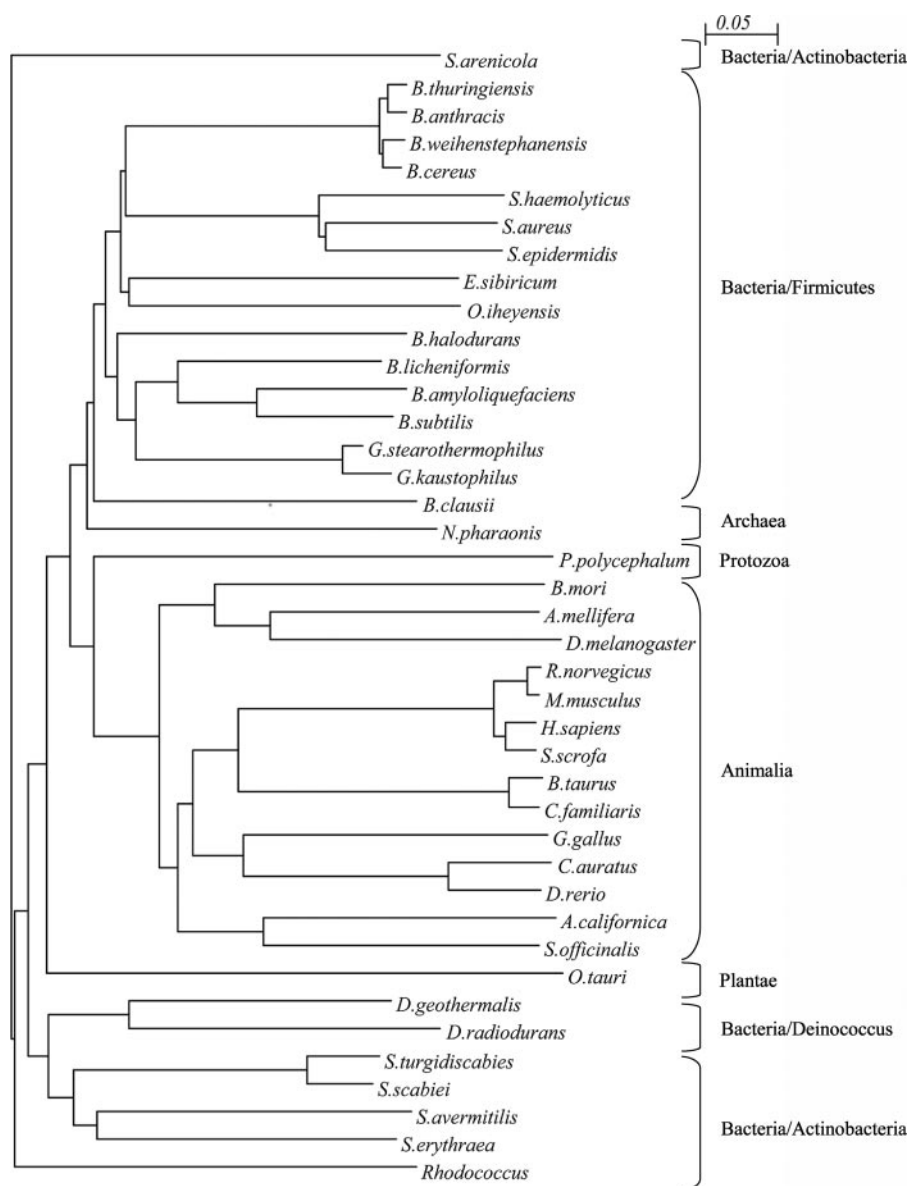


FIGURE 2. NOS evolution. Phylogenetic tree constructed based on the computational analysis of alignments of NOS-like genes across the whole NCBI data base.

TABLE 1

Strains used in this study

Strains	Relevant genotype	Reference
<i>B. subtilis</i>		
168 (WT)	<i>TrpC2</i>	Lab. collection
Δnos	<i>trpC2 nos::spc</i>	Lab. collection
<i>nasD</i> Δnos	<i>trpC2 nos::spc nasD::phleo</i>	This study
$\Delta cysJI$	<i>trpC2 cysJI::Cm</i>	This study
$\Delta ykuN$	<i>trpC2 ykuN::Cm</i>	This study
$\Delta ykuN$ Δnos	<i>trpC2 ykuN::Cm nos::spc</i>	This study
<i>bsNOS</i>	<i>trpC2 pUCND::nos</i>	This study
<i>nasD32</i>	<i>trpC2 pheA1 nasD::phleo pDH32::amyE</i>	This study
<i>baNOS</i>	<i>trpC2 pheA1 nasD::phleo pSYN::amyE</i>	This study
<i>nasD32</i> $\Delta kata$	<i>trpC2 pheA1 nasD::phleo pDH32::amyE kataA::spc</i>	This study
<i>baNOS</i> $\Delta kata$	<i>trpC2 pheA1 nasD::phleo pSYN::amyE kataA::spc</i>	This study
<i>nasD32</i> $\Delta soda$	<i>trpC2 pheA1 nasD::phleo pDH32::amyE sodaA::spc</i>	This study
<i>baNOS</i> $\Delta soda$	<i>trpC2 pheA1 nasD::phleo pSYN::amyE sodaA::spc</i>	This study
1972	<i>trpC2 pheA1 nasD::phleo</i>	M. Nakano
<i>E. coli</i>		
TOP10	<i>F⁻ mcrA Δ (mrr-hsdRMS-mcrBC) ϕ80lacZAM15 ΔlacX74 deoR nupG recA1 araD139 Δ (ara-leu)7697 galU galK rpsL(StrR) endA1 λ^-</i>	Invitrogen

other deletion strains. To make the *bsNOS* strain, the pUCND6 plasmid was transformed into the Δnos strain and chloramphenicol-resistant/spectinomycin-sensitive colonies were selected. Double cross-over recombination events were confirmed by PCR. To construct pUCND6, a PCR fragment containing the *araA* promoter and regulatory elements from *B. subtilis* was cloned in front of the *nos* gene in the pBNOS_{B.su} (I/V) plasmid. Then two 400-bp fragments upstream and downstream of *nos* (*yflM*) were amplified by PCR and cloned into the above plasmid upstream of the *araA* promoter and downstream of the *nos* gene. Finally, the chloramphenicol resistance gene from pDG268 was cloned between the *nos* gene and downstream fragment. To make *nasD32* and *baNOS* strains, pDH32 and pSYN plasmids, respectively, were transformed into the 1972 strain, and chloramphenicol-resistant colonies were selected. To construct pSYN, a PCR fragment containing the *nos* gene from *B. anthracis* (*banos*) was cloned into the pDH32 plasmid. Then the *araA* promoter and regulatory elements from *B. subtilis* were cloned upstream of the *banos* gene. To make *phmp-lacZ*, a PCR fragment containing the *hmp* promoter and regulatory elements from *E. coli* was cloned in front of the *lacZ* gene in pPROLarA/LacZ vector (Clontech Laboratories, Inc.).

Detection and Measurement of Bacterial NO Production—NO produced by cells is oxidized to nitrite and nitrate in aerated media. Hence, nitrite and nitrate concentrations directly correlate with the level of NO production. To measure nitrite and nitrate, cell culture supernatants were clarified by centrifugation and then filtered through YM-3 Microcons (Millipore). Nitrite and nitrate were measured in flow-through by the use of a fluorometric and colorimetric nitrate/nitrite assay kit (Cayman Chemicals). In the experiments with exogenous peroxide the H₂O₂ has been degraded before measurement of nitrate and nitrite. To remove H₂O₂ a small amount of MnO₂ was added to each sample and incubated for 20 min. NO production *in vivo* was detected by induction of *hmp* promoter-regulated *lacZ* expression and by the NO-specific, intracellular, fluorescent

CuFL probe as described (20, 21). Corresponding bacterial strains were grown to early exponential phase and then bNOS expression was induced by arabinose. Arginine was added to ensure substrate availability. In the case of *hmp* induction, detection samples were collected every hour for galactosidase activity assays (24). For NO detection by cell-permeable CuFL, the probe was prepared *in situ* just prior to use by mixing FL and CuCl₂ in a 1:1 ratio and then added to the growing bacterial cultures to a final concentration of 20 μ M. Twenty minutes to 1 h later, fluorescent and visible images of bacteria treated with the NO-detecting probe were taken by the digital camera attached to an Axio microscope (Carl Zeiss MicroImaging Inc.). The percentage of fluorescent bacteria was calculated by IPLab Scientific Image Processing software.

Nitrite and Nitrate Production by euNOS in Bacterial Cultures—Plasmids (pCWori) containing rat neuronal nNOSoxy domain (amino acids 1–720), full-length rat nNOS (amino acids 1–1570), or *B. subtilis* bsNOS DNAs were transformed into protease-deficient *E. coli* BL21(DE3). Overnight cultures grown in liquid LB medium were diluted in fresh LB, and cultures were grown at 37 °C with aeration until the A₆₀₀ reached 0.3. A solution containing 1 mM isopropyl- β -D-thiogalactoside, 450 μ M Δ -aminolevulinic acid, and 5 mM Arg was added to the culture. Bacterial cultures were harvested and processed for nitrite and nitrate measurements. Briefly, cultures were centrifuged and the supernatants were distributed into 96-well plates. Nitrite was detected by adding Griess reagent to each well and was quantified from the absorbance difference at 550 and 650 nm based on standard nitrite solutions (19). In some cases nitrite reductase was used to measure the total amount of nitrite plus nitrate produced. Similar measurements were performed using *E. coli* BL21 (DE3) containing no transformed plasmids as a control.

Alignment and Generation of NOS Tree—The *B. subtilis* NOS sequence was blasted against all sequences in the NCBI data base. All hits with sufficient score were downloaded and aligned by using ClustalX and NJplot software.

RESULTS

bNOS Produces NO in Vivo—Direct and accurate detection of NO in biological systems is a challenging task due to the very low concentrations and short life span of this free radical. To demonstrate bNOS-dependent NO production under physiological condition *in vivo*, we first relied on a quantitative assessment of the NO end products nitrate and nitrite. NO is a highly pervasive hydrophobic molecule that readily penetrates cell walls and membranes. For example, mammalian NO produced in endothelial cells quickly diffuses into neighboring smooth muscle cells to induce vasorelaxation. Once escaping from cellular confinement, NO is rapidly oxidized in the medium under aerobic conditions to form nitrite and nitrate. NO oxidation to nitrite and nitrate is also facilitated by transition metals and intracellular enzymes, *e.g.* by flavohemoglobin and truncated hemoglobin (25–29). Thus, the level of nitrite and nitrate in the medium indirectly reflects NOS activity.

We grew *B. subtilis* in rich medium and compared nitrite and nitrate production in the WT *versus nos* deletion strains. Total nitrite and nitrate concentration remained steady dur-

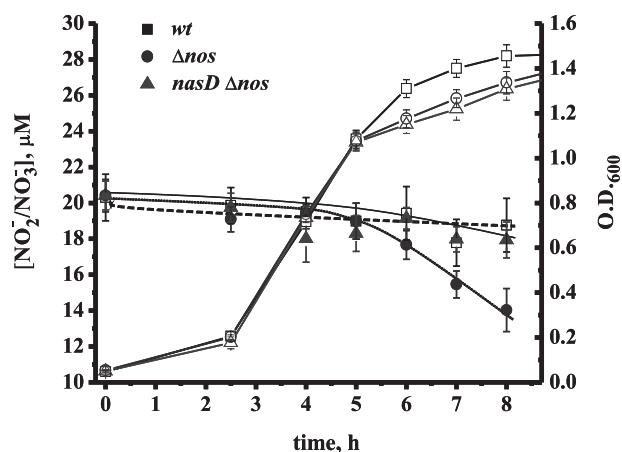


FIGURE 3. **bNOS-dependent NO production *in vivo*.** Effect of *nos* deletion on total nitrite and nitrate accumulation and growth rate of *B. subtilis*. WT and Δnos cells were grown in LB medium. Samples were collected every hour and nitrite and nitrate concentration determined in clarified supernatants. Data are shown as mean \pm S.E. from three experiments. Circles stand for WT, squares for Δnos mutant, and triangles for $\Delta nasD, \Delta nos$ double mutant cells. Open symbols stand for A_{600} (OD_{600}), closed for nitrite and nitrate concentration.

ing exponential growth and began to decrease upon transition into the stationary phase (Fig. 3). This decrease is due to nitrite and nitrate consumption during ammonification and can be abolished by deleting the *nasD* gene, which encodes for nitrite reductase (Fig. 3). However, in the WT strain the decrease was substantially less, indicating that bNOS-derived NO has been produced (Fig. 3). The amount of NO formed was not large, but very reproducible. A high output of NO cannot be expected because it is well known that persistently large concentrations of NO, such as produced by macrophage inducible NOS, is toxic to bacteria. Interestingly, the point at which the difference in nitrite and nitrate concentration between WT and Δnos becomes larger than experimental error occurs at the onset of the growth delay of Δnos (~ 6 h, Fig. 3), suggesting that bNOS activity is important for maintaining normal growth at the log-to-stationary transition phase.

Taken together, these results demonstrate that bNOS generates NO *in vivo* under physiological growth conditions. This NO is required for maintaining normal cell growth rate at the time of entering the stationary phase.

Rational Search for a bNOS Redox Partner—Because bNOS is functional *in vivo*, an intracellular reductase(s) that supports its activity must be acting *in trans*, serving as a specific or non-specific redox partner. Previously, we showed that the deletion of bNOS in *B. subtilis* sensitized cells to peroxide, implicating bNOS in a novel cell defense mechanism against oxidative stress (23). These results also provide us with a sensitive functional assay to monitor NOS activity *in vivo*.

The reductases YkuN and YkuP isolated from *B. subtilis* support NO synthesis by bNOS *in vitro* (19). We thus considered them promising candidates for a bNOS reductase *in vivo*. Deletion of the *ykuNP* genes did not sensitize *B. subtilis* to oxidative stress, however (Fig. 4). Another good candidate for a bNOS reductase is CisJ. This protein has the highest homology to the euNOS reductase domains and is not tethered to any redox

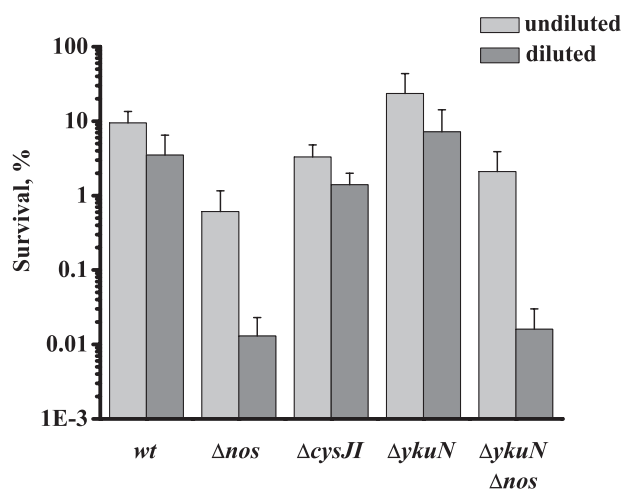


FIGURE 4. **Effect of *nos*, *cysJI*, and *ykuN* deletions on *B. subtilis* peroxide sensitivity.** Strains were grown aerobically in LB to late log phase. An aliquot from each culture was diluted with an equal amount of fresh prewarmed LB for 5 min (*diluted*). Both diluted and undiluted aliquots were treated with 10 mM H_2O_2 for 30 min; cells were plated on LB agar plates and colony-forming units counted on the next day. Values shown are means and S.D. (error bars) from three independent experiments.

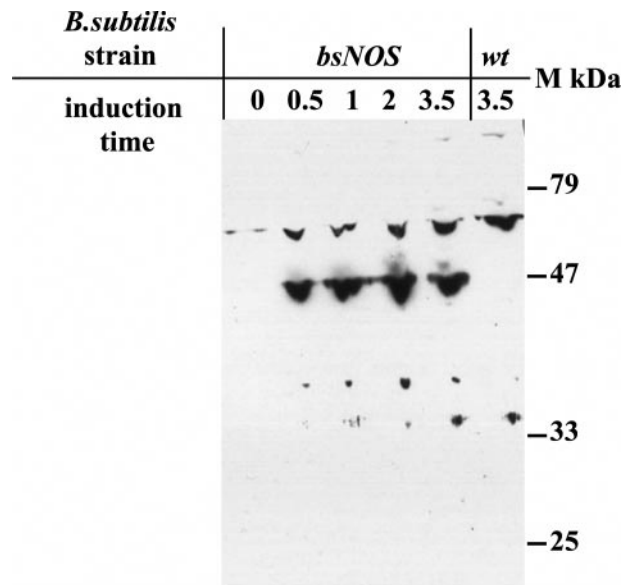


FIGURE 5. **Expression of bNOS in *B. subtilis* *bsNOS* strain.** Western blot analysis of bNOS expression in *B. subtilis* WT and *bsNOS* strains. Cells were grown in LB to $A_{600} \sim 0.5$, followed by arabinose and arginine addition. Samples were collected at the indicated time intervals and crude extracts resolved by SDS-PAGE. The membrane was stained by bNOS-specific antibodies.

partner. Yet again, deletion of *cisJ* did not alter *B. subtilis* sensitivity to peroxide in our test assay (Fig. 4). These results rule out the possibility that either YkuNP or CisJ is an indispensable bNOS redox partner and suggest that there may not be a bNOS-specific reductase. This notion was supported by our genomic analysis of bNOS in various bacteria. It is well established that bacterial genes encoding multisubunit enzymes are usually clustered in operons (30). Using a BLAST search we found *nos* genes in at least 22 bacterial species. None of these genes was located in the same operon with any known reductase or reductase-like protein (supplemental Table S1). We therefore conclude that no specific bacterial reductase exists to support NO synthesis by bNOS.

NO Synthesis in Bacilli

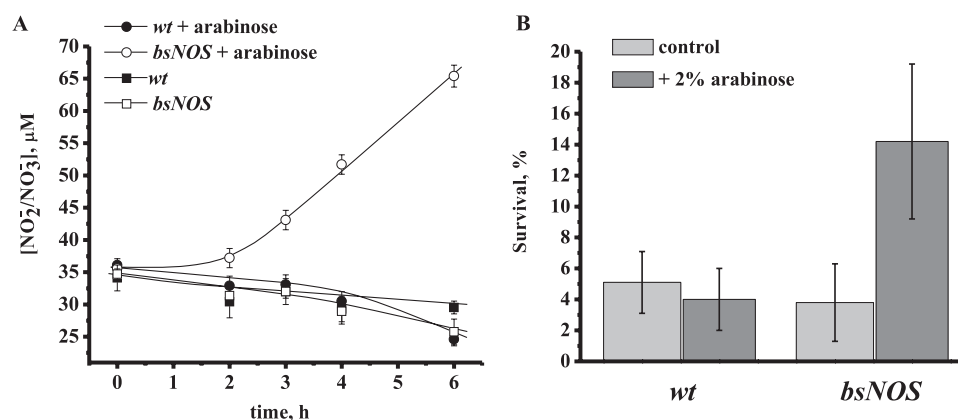


FIGURE 6. NO production as a function of bNOS expression in *B. subtilis*. A, monitoring nitrite and nitrate accumulation in response to bNOS overexpression. *B. subtilis* WT and *bsNOS* cells were grown in LB to $A_{600} \sim 0.5$, followed by arabinose and arginine addition. Samples were collected every hour and nitrite and nitrate detected in the supernatant. Data are shown as mean \pm S.E. from four experiments. B, induction of peroxide resistance by bNOS-mediated NO synthesis. WT and *bsNOS* strains were grown in LB medium to $A_{600} \sim 0.3$, followed by arabinose and arginine addition. After 1 h of incubation, bacteria were challenged with 10 mM H_2O_2 for 30 min. The percentage of surviving cells was determined by colony formation and is shown as the mean \pm S.D. from three experiments.

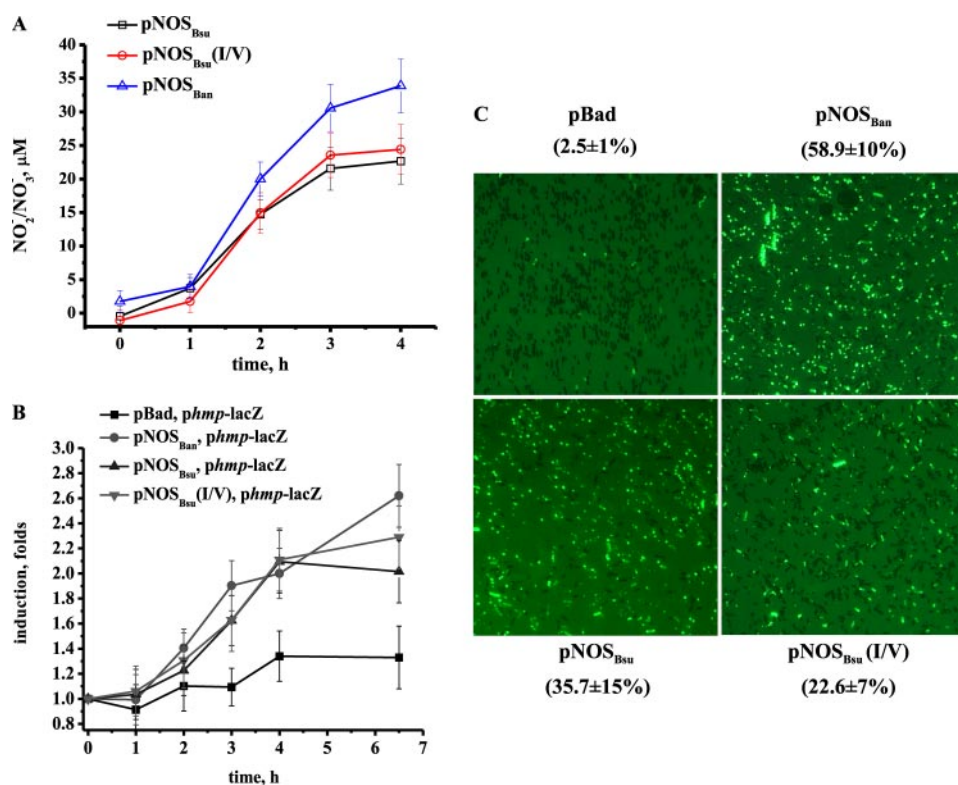


FIGURE 7. Successful bNOS transplantation from *Bacilli* to *E. coli*. A, nitrite and nitrate accumulation in *E. coli* culture in response to bNOS expression. The plot shows normalized nitrite and nitrate levels in supernatants of *E. coli* strains harboring pBAD plasmids with WT bNOS from *B. anthracis* (pNOS_{Ban}), WT bNOS from *B. subtilis* (pNOS_{Bsu}), and mutant bNOS with an increased activity from *B. subtilis* (pNOS_{Bsu}(I/V)). Experimental conditions are as in Fig. 6A. All values were normalized against the empty vector control. Mean \pm S.D. from four experiments. B, induction of *E. coli* hmp promoter in response to bNOS expression. Experimental conditions are as in A except that all cells contained a pBAD-compatible plasmid expressing a lacZ reporter under the hmp promoter. Induction was calculated based on the Miller unit change. Mean \pm S.D. from three experiments. C, representative fluorescent image of bacteria treated with the Cu(II)-based NO-detecting probe (CuFL). Experimental conditions are as in A. Pictures were taken 1 h after CuFL addition. The percent of fluorescent cells (mean \pm S.D.) is indicated for each strain.

bNOS Is Limited with Respect to a Pool of Available Redox Partners—Previous work reveals that reductases need not be dedicated to any specific enzyme. For example, in *Streptomyces* one reductase serves as an electron donor for 17 dif-

ferent cytochromes P450 (31). Bacterial genomes contain numerous reductase-like proteins. In *B. subtilis* ~ 90 reductases have been annotated, with 40 of them having no known function. We thus rationalized that if *B. subtilis* NOS has no dedicated redox partner, reductase activity is unlikely to be the rate-limiting step in the enzymatic pathway leading to NO production. In other words, available reductases are expected to be present in excess over bNOS. To test this prediction, we examined the level of NO as a function of bNOS concentration by substituting an authentic bNOS promoter with a strong arabinose-inducible promoter (pAra) in a chromosome. Arabinose-dependent bNOS expression was confirmed by Western blotting using bNOS-specific antibodies (Fig. 5). As Fig. 6A shows, the addition of arabinose caused the nitrite and nitrate level to rise dramatically in the pAra-bNOS-expressing strain, but not in a WT control.

Because NO protects bacteria from oxidative stress (23), we also tested whether pAra-bNOS expression makes cells more resistant to peroxide. As expected, arabinose rendered *BsNOS* cells five times more resistant to 10 mM H_2O_2 than control cells (Fig. 6B). Moderate level of protection correlates well with slow but continuous (~ 120 nm/min) NO synthesis by *BsNOS* cells. These results confirm the ability of bNOS to produce NO efficiently and also demonstrate that it is limiting with respect to available cellular reductases that support its activity.

bNOS Is Functional in a Foreign Bacterial Host—The absence of any specific redox partner suggests that bNOS can be functional even in those bacterial species that have lost or not evolved the ability to produce NO aerobically because they could still have nonspecific reductases to

assist bNOS. We chose *E. coli* as a recipient for such a transplantation experiment. Gram-negative *E. coli* does not have nos-like genes and is very distant in evolution from Gram-positive *Bacilli*.

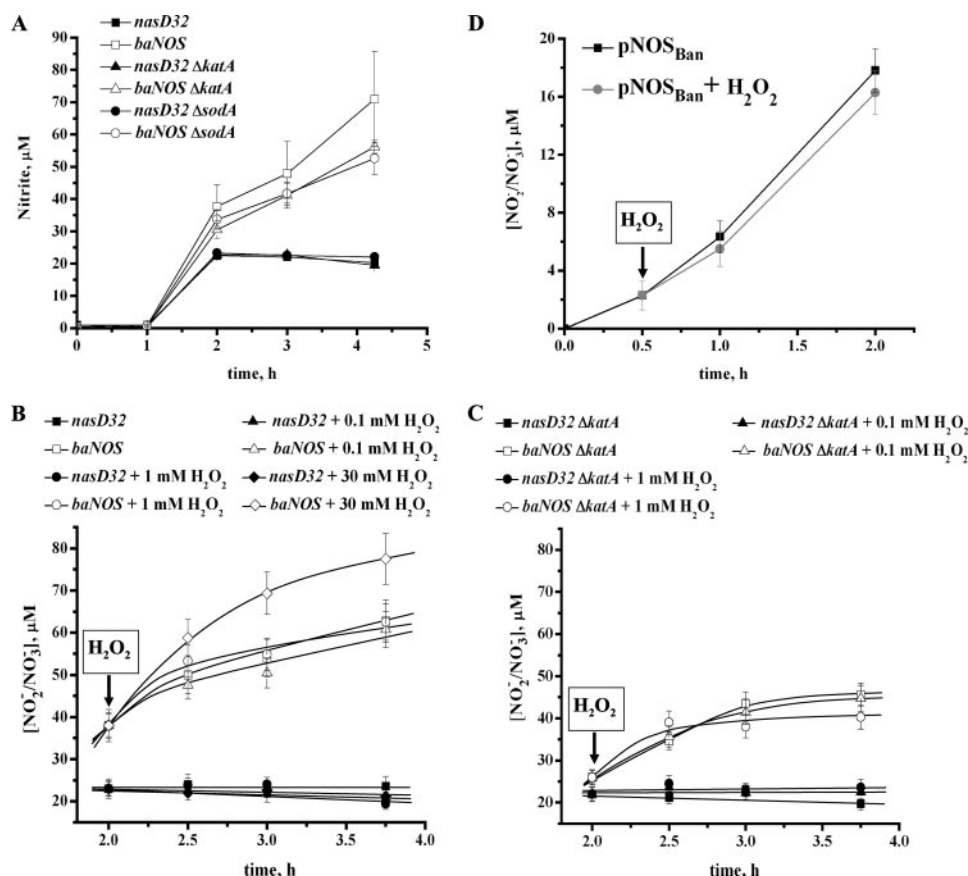


FIGURE 8. Effect of peroxide or superoxide on NO production. Monitoring nitrite and nitrate accumulation in response to elevated endogenous peroxide or superoxide level (A) and after H_2O_2 addition to the WT (B) and *katA*-deficient (C) *B. subtilis* strains. Strains were grown in LB to $A_{600} \sim 0.3$, followed by arabinose addition (time 0) to induce baNOS expression. Hydrogen peroxide was added 2 h later. Samples were collected every half hour; excess of H_2O_2 was removed by treatment with MnO_2 and nitrite and nitrate detected in the supernatant. Open symbols stand for baNOS-expressing strains, closed for the parental strain. Data are shown as mean \pm S.E. from three experiments. D, effect of exogenous peroxide on NO production in *E. coli*. The strain expressing baNOS ($p\text{NOS}_{\text{Ban}}$) was grown in LB to $A_{600} \sim 0.3$, followed by arabinose and arginine addition (time 0) to induce baNOS expression. Hydrogen peroxide (1 mM) was added 30 min later. Samples were collected and nitrite and nitrate detected in the supernatant. Data are shown as mean \pm S.E. from three experiments.

Three different bNOS versions were cloned into a pBAD expression vector. The resulting plasmids $p\text{NOS}_{\text{Bsu}}$, $p\text{NOS}_{\text{Ban}}$, and $p\text{NOS}(\text{I/V})_{\text{Bsu}}$ expressed WT bNOS from *B. subtilis*, WT bNOS from *B. anthracis*, and mutant bNOS from *B. subtilis*, respectively. The last exhibits increased activity *in vitro* due to Ile to Val substitution near the heme pocket (32). Induction of each of these plasmids in an *E. coli* led to increased media levels of nitrite and nitrate (Fig. 7A). Consistently, the I/V bNOS mutant produced slightly more nitrite and nitrate than WT bNOS. Curiously, bNOS from *B. anthracis* was the most active as judged by the amount of accumulated nitrite and nitrate. These results demonstrate that bNOS can efficiently hijack an available reductase even from a foreign host to produce NO.

To confirm that the expression of bNOS in *E. coli* generates NO, not just nitrite and nitrate, we took advantage of the pre-existing intracellular sensor of NO in *E. coli*. Transcription of the flavohemoglobin gene (*hmp*) is specifically up-regulated by NO in both *B. subtilis* and *E. coli* (33–36). To monitor *hmp* response to endogenous NO, we made a transcriptional fusion of the *lacZ* reporter with the *hmp* promoter. As Fig. 7B shows, the expression of bNOS led to the increased galactosidase activ-

ity in *hmp-lacZ* fusion cells. The rates of *hmp* induction observed in this experiment correlate well with a slow continuous NO production and contrast the sharp and brief induction in response to bolus treatment using relatively fast releasing NO donors (33–35, 37).

Finally, we utilized the NO-specific fluorescent probe CuFL, which has only recently become available (21), to monitor directly NO production by bNOS in *E. coli*. In all bNOS-expressing strains cells turned bright green upon treatment with CuFL (Fig. 7C). In the empty vector control, however, only a few bacteria cells became fluorescent (Fig. 7C), which may correspond to very low nonspecific reactivity of the probe. Curiously, fluorescence tended to concentrate at the poles of bacterial cells, indicating a preferred localization of either the bNOS enzyme or the probe.

DISCUSSION

Our results demonstrate that bNOS efficiently produces NO *in vivo* and does so without any reductase domain or dedicated subunit. Instead, it utilizes an available cellular redox partner to support its NO synthesis. Alternatively, bNOS may utilize small molecules to reduce iron in its active site.

Intracellular cysteine, for example, is capable of reducing cellular iron and is present in a sufficiently high concentration to do so in the bacterial cell (23). However, we found that cysteine does not support NO synthesis by bNOS *in vitro*, even at levels much greater than physiological (data not shown).

Hydrogen peroxide at high concentrations (30 mM) supports NO synthesis by bNOS *in vitro* (9, 32). To verify that physiological levels of peroxide or superoxide can support NO synthesis we expressed the *nos* gene in a $\Delta katA$ or $\Delta sodA$ background. KatA is the major vegetative catalase in *B. subtilis*, which accounts for $\sim 98\%$ of peroxide scavenging activity (38). SodA supplies all superoxide-scavenging activity in *B. subtilis* (39). Thus, in these strains the intracellular level of peroxide (in $\Delta katA$) or superoxide (in $\Delta sodA$) should be elevated. However, neither deletion resulted in increased NO production (Fig. 8A). To verify that the peroxide does not stimulate bNOS activity *in vivo* under physiological conditions we examined the effect of exogenous peroxide on NO production. Fig. 8B shows that exogenous peroxide is capable of boosting NO production *in vivo* only at 30 mM; at lower concentrations H_2O_2 cannot drive the reaction. This result is consistent with previous *in vitro* data

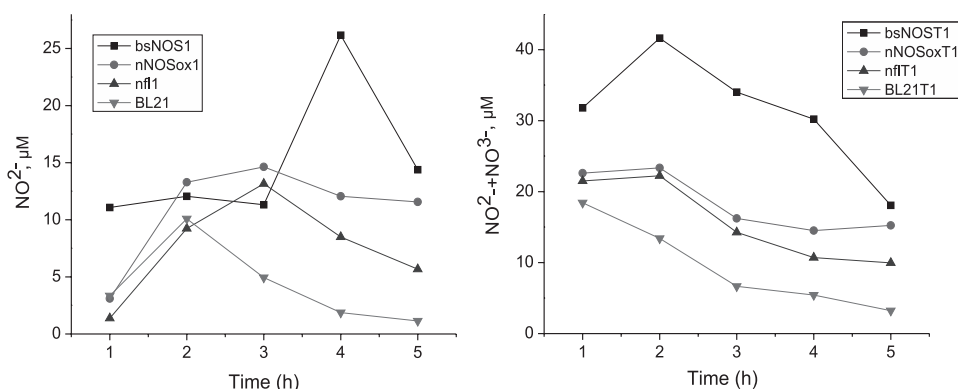


FIGURE 9. **The oxygenase domain of mammalian NOS is functional in *E. coli*.** Time-dependent nitrite (left) and the sum of nitrite and nitrate (right) formation in *E. coli* cultures expressing the following proteins: *bsNOS*, NOS from *B. subtilis*; *nNOS_{oxy}*, oxygenase domain of rat nNOS; *nfl*, full-length rat nNOS. BL21 stands for control cells that did not express any protein. Data points are the mean of triplicate determinations and are representative of two independent experiments.

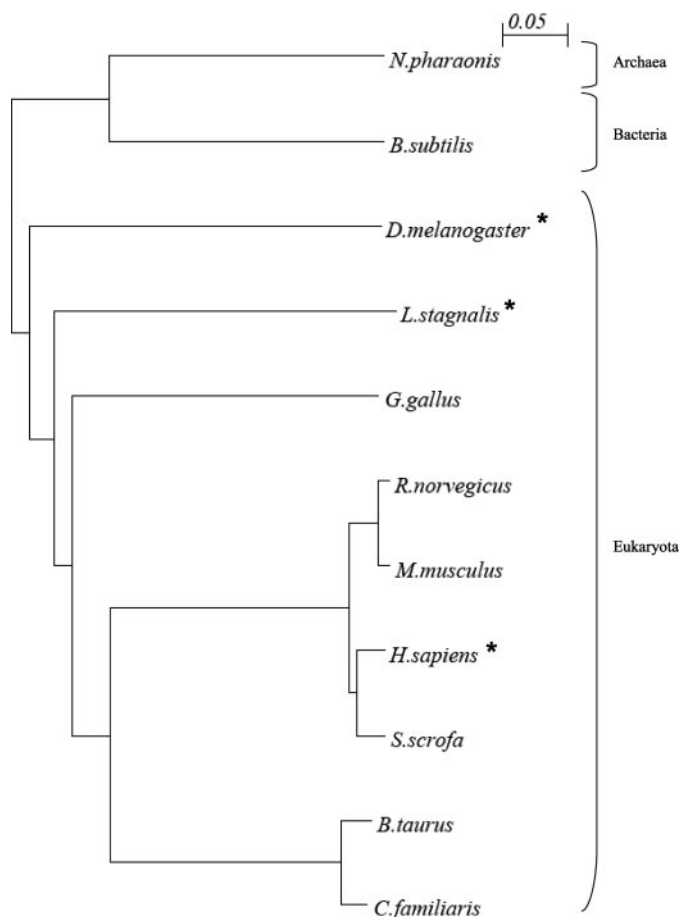


FIGURE 10. **Truncated NOSes from simple eukaryotes as an intermediate step in NOS evolution from bacteria.** Phylogenetic tree constructed based on the computational analysis of alignments of NOS-like genes. The organisms in which the truncated forms of NOS were detected are marked with asterisks.

(9, 32). Obviously, such a huge amount of peroxide cannot be achieved *in vivo*. Similar results were obtained using the catalase-deficient *B. subtilis* strain (Fig. 8C), which excludes the possibility of rapid peroxide degradation, or bNOS-expressing *E. coli* strain (Fig. 8D). We therefore conclude that bNOS relies on a protein redox partner rather than a small molecule reductant.

Many reports show that alternative transcription initiation and splicing of euNOS (e.g. in human, mice, snail, and fruit fly) lead to formation of proteins lacking the carboxyl-terminal reductase domain (40–42). It has been proposed that such truncated polypeptides participate in posttranslational regulation of NOS activity. However, their ability to produce NO has neither been verified nor discussed. Because bNOS activity is supported by non-specific reductases, we decided to examine the possibility that mammalian carboxyl-terminal reductase domain-deficient NOS can also

generate NO in bacteria by hijacking available reductases. We cloned the full-length rat nNOS and its oxygenase domain (nNOS_{oxy}) in the *E. coli* expression vector and measured nitrite and nitrate production. As predicted, induction of both nNOS and nNOS_{oxy} expression resulted in the marked elevation of nitrite and nitrate in the medium (Fig. 9), indicating that bacterial reductases can efficiently donate electrons to mammalian NOS expressed in bacteria for NO synthesis.

NO functioning has been greatly diversified and specialized during evolution. With the appearance of multicellular organisms, NO became progressively involved in intracellular and intercellular signaling. Acquisition of reductase and calmodulin-binding domains by euNOS rendered it more efficient and amenable to regulation by calmodulin, which is activated by increased levels of Ca²⁺. Because multiple truncated versions of euNOS could be detected in fruit flies and snails (40, 42), it is reasonable to speculate that these organisms have the earliest forms of the full size euNOS that, however, still share their function with more primitive forms (Fig. 2). Our phylogenetic analysis also supports this hypothesis (Fig. 10).

From a practical point of view, our work suggests a novel approach for a controlled and continuous delivery of physiological amounts of NO for research and medical purposes. Indeed, NO-producing bacteria can be regarded as live NO donors, for which the rate of NO production can be easily set to a desired value or regulated during bacterial growth. Many pathological conditions are associated with compromised NO production (3, 43). Hence, numerous chemical NO donors have been developed to meet this medical need. However, most available NO donors are unstable in physiological vehicles. They decompose with unpredictable rates *in vivo* and release NO too quickly. These and other unrelated toxicity issues have kept most NO donors from clinical use. In contrast, probiotic bacteria can be designed to constantly generate NO over days with the rates adjusted to the host needs. Such bacteria-derived NO generated in the gastrointestinal tract will be quickly absorbed by circulating blood and distributed to distant tissues in the more stable form of S-nitrosothiols (44, 45). Not only non-pathogenic *bacilli*, but also many other probiotic bacteria, could be used for this purpose. Future studies will show whether such an approach is viable.

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